Research Article

Proteomic analysis of *Bacillus subtilis* strains engineered for improved production of heterologous proteins

Susanne Pohl[1], Gaurav Bhavsar[2], Joanne Hulme[2], Alexandra E. Bloor[2], Goksel Misirli[1,3], Matthew W. Leckenby[2], David S. Radford[2], Wendy Smith[1,3], Anil Wipat[1,3], E. Diane Williamson[4], Colin R. Harwood[1] and Rocky M. Cranenburgh[2]

[1] Centre for Bacterial Cell Biology, Baddiley-Clark Building, Newcastle University, Newcastle upon Tyne, UK
[2] Cobra Biologics, Stephenson Building, Keele Science Park, Keele, Staffordshire, UK
[3] Computing Science, Claremont Tower, Newcastle University, Newcastle upon Tyne, UK
[4] Biomedical Sciences, Defence Science and Technology Laboratory, Porton Down, Salisbury, Wiltshire, UK

The use of bacterial systems for recombinant protein production has advantages of simplicity, time and cost over competing systems. However, widely used bacterial expression systems (e.g. *Escherichia coli*, *Pseudomonas fluorescens*) are not able to secrete soluble proteins directly into the culture medium. This limits yields and increases downstream processing time and costs. In contrast, *Bacillus* spp. secrete native enzymes directly into the culture medium at grams-per-litre quantities, although the yields of some recombinant proteins are severely limited. We have engineered the *Bacillus subtilis* genome to generate novel strains with precise deletions in the genes encoding ten extracytoplasmic proteases that affect recombinant protein secretion, which lack chromosomal antibiotic resistance genes. The deletion sites and presence of single nucleotide polymorphisms were confirmed by sequencing. The strains are stable and were used in industrial-scale fermenters for the production of the *Bacillus anthracis* vaccine protein, protective antigen, the productivity of which is extremely low in the unmodified strain. We also show that the deletion of so-called quality control proteases appears to influence cell-wall synthesis, resulting in the induction of the cell-wall stress regulon that encodes another quality control protease.

Received: May 13, 2013
Revised: July 25, 2013
Accepted: August 21, 2013

Keywords:
Anthrax protective antigen / Biomedicine / Gene deletion / Proteases / Recombinant protein / Secretion

Additional supporting information may be found in the online version of this article at the publisher's web-site

1 Introduction

Bacteria have been used for decades as sources of industrial and research enzymes, therapeutic proteins and metabolites, including antibiotics, food additives and nutritional supplements. Members of the genus *Bacillus* in particular are prodigious producers of industrial enzymes, reflecting their ability to breakdown and utilise soil detritus (nucleic acids, proteins, starch, pectin, cellulose, etc.) [1, 2]. The ability of *Bacillus subtilis* and its close relatives to secrete proteins directly into the culture medium at high concentrations provides a more cost-effective means of producing proteins than extraction from the cytoplasm or periplasm. Moreover, secreted proteins often exhibit improved structural authenticity and significantly less processing is required to avoid the co-purification of undesirable contaminants such as endotoxins and nucleic acids. Given these potential advantages, it is surprising that *Bacillus*

Correspondence: Dr. Rocky M. Cranenburgh, Cobra Biologics, Stephenson Building, Keele Science Park, Keele, Staffordshire, ST5 5SP, UK
E-mail: rocky.cranenburgh@cobrabio.com
Fax: +44-1782-799 817

Abbreviations: CWBP, cell wall binding proteins; **MGT**, mean generation time; **rPA**, recombinant protective antigen; **SNP**, single nucleotide polymorphism; **WapA**, wall-associated protein A

© 2013 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

species are not more widely used for the production of heterologous proteins. In practice, attempts to use this group of bacteria for the manufacture of heterologous proteins have met with mixed success [3]. The reasons are complex, but relate to intrinsic properties of both the secretion system and the target proteins themselves. Two well-established bottlenecks are a series of quality control checkpoints designed to avoid potentially lethal blockages of the secretion pathway and cell-wall synthesis, and the secretion of non-discriminatory "feeding" proteases that allow the cells to recover amino acids and peptides from proteins in the environment [4].

More recently, microorganisms have been seen as sources of environmentally compatible alternatives for a wide range of otherwise energy inefficient and polluting industrial processes. One widely used approach to strain development is to minimise the genomes of established industrial microorganisms, and such an approach has been applied to a range of microbes including *B. subtilis*, *Clostridium glutamicum*, *Escherichia coli*, *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* [5]. Genome reduction seeks to develop strains with characteristics that are more favourable for industrial processes by addressing the question of what is the minimal set of genes required for the efficient conversion of substrate to product in an industrial bioreactor. While such programmes have been successful in deleting large sections of the *B. subtilis* genome [6–8], there are few reports concerning the characteristics of these strains in an industrial setting.

Here, we describe a systematic approach to strain optimisation involving the removal of bottlenecks for the production of heterologous proteins by *B. subtilis*. In contrast to the genome minimalisation approach, we have targeted the removal of specific genes known or suspected to affect the stability of heterologous proteins released into the culture medium. Using the anthrax protective antigen (PA) as a model heterologous protein, which normally accumulates at very low concentrations in the culture medium, proteomics was used to assess the performance of the resulting strains in the laboratory and an industrial bioreactor. The results show the impact of the proteases deletions, not only on the yield of PA but also on cell-wall stress.

2 Materials and methods

2.1 Construction of protease gene deletion mutants

The parental strain, *B. subtilis* 168 (*trpC2*) [9], was obtained from the Institut Pasteur Culture Collection (CIP106309). To create the gene deletion loci for *nprB*, *aprE*, *epr* and *bpr*, the genes and flanking regions were amplified from chromosomal DNA using the proximal and distal primers suffixed '5XBA' and '3XBA' (Supporting Information Table 1). These PCR products were used as templates to amplify the 5′ and 3′ flanks of the genes that were joined using splicing PCR and the homology between the 5′ ends of the internal primers (suffixed 'XHO'). In the case of *nprB*, the flanking PCR products were cut at introduced XhoI restriction sites and ligated. The deletion loci for *nprB*, *aprE*, *epr* and *bpr* were cloned into pORI240 [10], generating plasmids pORIΔnprB, pORIΔaprE, pORIΔepr and pORIΔbpr. pORI240 facilitates unlabelled gene deletions and diagnostic PCR was used to identify the required deletion mutants.

Gene deletions were isolated by transforming [11] *B. subtilis* with derivatives of pORI240. The gene deletions were generated in the order of *nprB*, *aprE*, *epr* and *bpr*, resulting in quadruple protease deletion mutant BRB04. However, attempts to delete *mpr* and *nprE* using the pORI240-based system proved to be impossible as the resolution of the integrated plasmid invariably generated wild-type rather than mutant genotypes, and consequently we switched to the Xer-cise system [12]. The *mpr* and *nprE* deletion plasmids (pmpr-DifCAT and pnprE-DifCAT) were used to delete *nprE* and then *mpr* from BRB04 as described above to create BRB06. To delete *vpr*, the primers SacTF and ywcHR were used to amplify *vpr* and its flanking regions and this PCR product was cloned into plasmid pCR2.1-TOPO. The resulting plasmid, pTOPO-vpr, was cut with NdeI (then blunted) and BspDI, and the *dif*-flanked chloramphenicol resistance gene (excised using HpaII and BfrBI) from pTOPO.bac-DifCAT [12] was ligated in to create the knockout plasmid pvpr-DiFCAT. This was linearised and used to transform BRB06 to generate BRB07, a strain with all the extracellular proteases deleted.

Deletion cassettes for *wprA*, *htrA* and *htrB* were synthesised and supplied in plasmid pPCR-Script (Geneart, Germany) with two 200 bp flanking regions separated by 18 bp containing AgeI and NsiI sites (Supporting Information Table 2). The *dif*-flanked chloramphenicol resistance gene from pTOPO.bac-DifCAT was then excised using AgeI and NsiI and cloned into the AgeI and NsiI sites of each deletion loci plasmid to create the Xer-cise deletion plasmids (pDwprA-DifCAT, pDhtrA-DifCAT, pDhtrB-DifCAT). The final mutant strain with ten genes deleted, BRB14, was generated from BRB07 by sequential deletion of *wprA*, *htrA* and *htrB* using these plasmids (Fig. 1). Additionally, a strain with nine genes deleted, BRB13, was created that retained a functional *wprA*. All deletion loci were confirmed by DNA sequencing (see Fig. 1 and Supporting Information Table 3).

2.2 DNA sequencing

Primers were designed to confirm the sequences associated with the loci of each deleted protease gene following their construction (Supporting Information Table 1). In addition, the entire genome of BRB14 was sequenced using an ion PGM^{TM} sequencer and Ion 316 chip (Life Technologies Ltd., UK). The DNA sequence of *B. subtilis* strain 168 was used for assembly and comparison [9].

© 2013 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

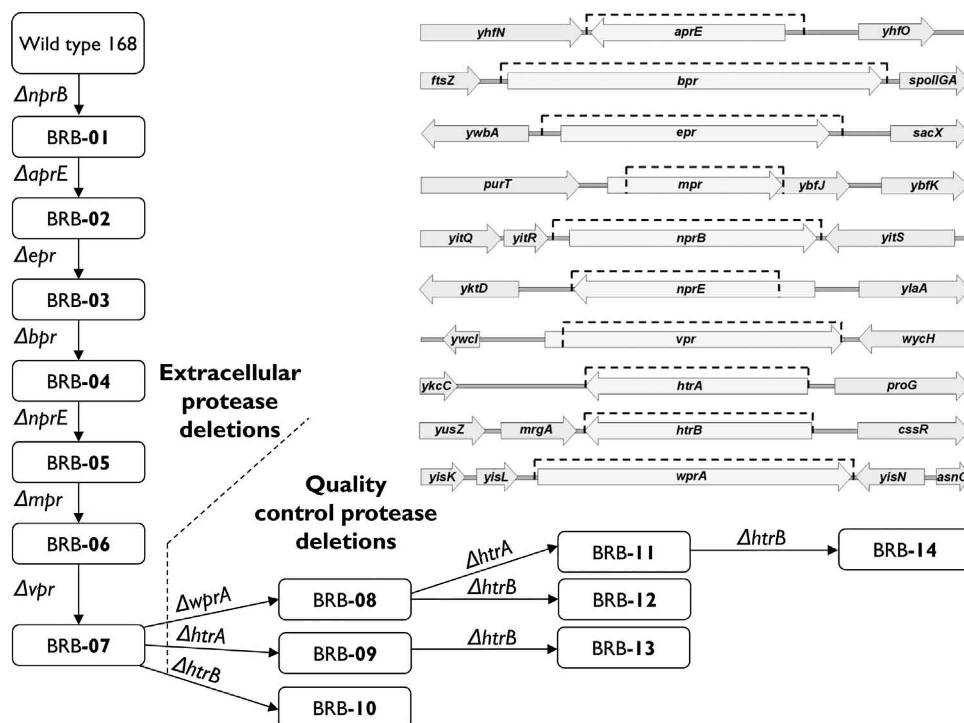

Figure 1. The protease deletion strains were constructed sequentially as shown in this figure. The wild-type loci are displayed in the inset, with the protease genes and 1 kb of flanking homology with flanking genes. A dashed line indicates the regions deleted in the protease-deficient strains.

2.3 Construction of PA expression plasmid

The *Bacillus anthracis* recombinant PA (rPA) gene (*pagA*) was cloned from pPA101–1 [13]. The expression plasmid used to secrete rPA was based on pHT01 (Mobitec, Germany) in which *pagA* expression was controlled from a P_{grac} promoter (a hybrid P_{groEL}–P_{lac} promoter). Firstly, a sequence containing the 28-residue signal peptide for the *B. licheniformis* α-amylase (AmyL) was synthesised with a 3′ Pst1 site and cloned into the BamHI-XbaI sites of pHT01. The *pagA* gene was amplified in two separate PCRs using primer combinations S-pag28-1/AS-pag28-1 and S-pag28-2/AS-pag28-2 to remove an internal PstI site (Supporting Information Table 1). The PCR products were then cloned into the PstI-XbaI-cut plasmid to create pHT28pagA. This was transformed into the six *B. subtilis* strains described below following the method in Bloor and Cranenburgh [12].

2.4 Expression of PA

The secretion of rPA from *B. subtilis* was studied using pHT28pagA-encoding strains 168, BRB07, BRB08, BRB11, BRB12 and BRB14. Strains were initially cultured overnight in 200 mL Erlenmeyer flasks in Terrific Broth containing 10 μg/mL chloramphenicol at 37°C and 200 rpm and then inoculated into 5.0 L fermenters to an optical density (OD_{600nm}) of 0.3–0.4. The medium comprised 4.0 L Spizizen's minimal medium, trace elements [11], chloramphenicol (10.0 μg/mL), tryptophan (40.0 μg/mL), potassium glutamate (1.0 g/L), glucose (4.0 g/L) and 2 mL Simethicone Antifoam PD30 (Basildon Chemical Co. Ltd., UK). Fermentation proceeded at 37°C, maintaining an O_2 concentration of 30% and a pH 7.0. Cultures were induced at OD_{600nm} = 10.0–14.0 using 1.0 mM IPTG and were then batch-fed with glucose (600 g/L), yeast extract (18.75 g/L) and phytone peptone (18.75 g/L) to maintain a constant growth rate. Samples were taken at hourly intervals following induction, centrifuged to generate a cell pellet and the cell-free supernatant was extracted, frozen and stored at −80°C. The supernatant samples were thawed and 16 μL subjected to PAGE (12% acrylamide/bis-acrylamide in MOPS buffer). The gel was then blotted onto a nitrocellulose membrane, probed with an anti-PA antibody, then a secondary alkaline phosphatase-conjugated rabbit anti-mouse antibody (AbCam, UK), and developed using NBT/BCIP solution (Sigma-Aldrich, USA).

2.5 Proteomics and MS

Extracellular protein extracts were prepared by harvesting cells (100 mL) by centrifugation at 10 000× *g* (40 min, 4°C). The extracytoplasmic proteins in the supernatant fraction were precipitated with ice-cold 10% w/v trichloroacetic acid overnight and prepared for proteome analysis as described previously [14]. The concentration of the protein samples was determined using the 2D Quant kit (GE Healthcare, UK). Protein samples (300 μg) were analysed by 2DE using IPG strips (GE Healthcare) in the pH range 3–10 for separation in the first dimension [15]. The gels were stained with Coomassie

© 2013 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Table 1. *B. subtilis* 168 strains generated during this study

Strain	Genotype	Deletion plasmid
BRB01	*trpC2, nprB*	pORIΔnprB
BRB02	*trpC2, nprB, aprE*	pORIΔaprE
BRB03	*trpC2, nprB, aprE, epr*	pORIΔepr
BRB04	*trpC2, nprB, aprE, epr, bpr*	pORIΔbpr
BRB05	*trpC2, nprB, aprE, epr, bpr, nprE*	pnprE-DifCAT
BRB06	*trpC2, nprB, aprE, epr, bpr, nprE, mpr*	pmpr-DifCAT
BRB07	*trpC2, nprB, aprE, epr, bpr, nprE, mpr, vpr*	pvpr-DifCAT
BRB08	*trpC2, nprB, aprE, epr, bpr, nprE, mpr, vpr, wprA*	pDwprA-DifCAT
BRB09	*trpC2, nprB, aprE, epr, bpr, nprE, mpr, vpr, htrA*	pDhtrA-DifCAT
BRB10	*trpC2, nprB, aprE, epr, bpr, nprE, mpr, vpr, htrB*	pDhtrB-DifCAT
BRB11	*trpC2, nprB, aprE, epr, bpr, nprE, mpr, vpr, wprA, htrA*	pDhtrA-DifCAT
BRB12	*trpC2, nprB, aprE, epr, bpr, nprE, mpr, vpr, wprA, htrB*	pDhtrB-DifCAT
BRB13	*trpC2, nprB, aprE, epr, bpr, nprE, mpr, vpr, htrA, htrB*	pDhtrB-DifCAT
BRB14	*trpC2, nprB, aprE, epr, bpr, nprE, mpr, vpr, wprA, htrA, htrB*	pDhtrB-DifCAT

blue (PlusOne Coomassie tablets, GE Healthcare) and the images were analysed using the Delta2D software (Decodon GmbH, Germany). Protein spots were subjected to in-gel tryptic digestion [16] and the resulting peptide digests were analysed using a Voyager DE-STR MALDI-TOF mass spectrometer (Applied Biosystems, Inc., USA). The instrument was equipped with a delayed extraction ion source, used a nitrogen laser at 337 nm and was operated in reflector mode at accelerating voltages of 20–25 kV. Mass spectra were typically obtained over a mass range of 900–4000 Da and monoisotopic peptide mass fingerprints were assigned and used for database searches. Identifications were performed using the peptide mass fingerprint data and the Mascot search engine program (Matrix Science Ltd., UK) where the peptide mass tolerance was limited to 50 ppm, searched against the MSDB protein sequence database.

3 Results

3.1 Generation of multiple protease deletions

Bacillus subtilis encodes three proteases (HtrA, HtrB and WprA) that are known to be functional at the wall/membrane interface or in the wall itself (quality control proteases), and seven (AprE, Bpr, Epr, Mpr, NprB, NprE and Vpr) that are secreted into the culture medium (feeding proteases). Previous work has shown that some or all of these proteases are responsible for the reduced yields of various heterologous proteins [17–19]. Previous attempts to inactivate combinations of these proteases were primarily generated for experimental purposes, and therefore the resulting strains were not constructed under conditions required for regulatory approval for the production of therapeutic proteins since the sources of the strains are unknown, the mutations poorly characterised and often include antibiotic resistance genes. We, therefore, constructed a series of mutants of an authenticated *B. subtilis* 168 strain in which each of the genes encoding the extracytoplasmic proteases was deleted using a precise nucleotide-to-nucleotide excision approach (Fig. 1). The *nprB*, *aprE*, *epr* and *bpr* genes were deleted using pORI240 [8], which generates unlabelled scarless gene deletions, while the *nprE*, *mpr*, *vpr*, *wprA*, *htrA* and *htrB* were deleted using the Xer-cise system [12], which leaves a 28 bp *dif* sequence scar (Supporting Information Table 3).

The strains with deletions in the ‘feeding’ proteases were generated sequentially in the order *nprB*, *aprE*, *epr*, *bpr*, *nprE*, *mpr*, *vpr* (Table 1). In each case, the deletion vector was introduced by selecting for the appropriate antibiotic resistance genes, and then made markerless, either by selecting for resistance to sucrose toxicity (pORI240-based vectors) or isolating antibiotic sensitive clones generated spontaneously after overnight incubation (DifCAT-based vectors). The authenticity of the deletions in the resulting strain, BRB07, was confirmed by sequencing over each of the deletion sites (Supporting Information Table 3).

The three quality control proteases, WprA, HtrA and HtrB, scan secretory proteins at the membrane/wall interface and in the wall for structural authenticity [3, 19–21]. Misfolded or slowly folding proteins are rapidly degraded to prevent interference with cell-wall growth [4, 20]. Although the genes encoding these proteases can be deleted individually without major effects on cell physiology [21, 22], strains in which both the *htrA* and *htrB* genes were deleted exhibit a significant reduction in viability and are prone to accumulating suppressor mutations [22]. We, therefore, used BRB07 as the starting point for a series of strains (BRB08, BRB09 and BRD10) in which additional single deletions were generated in *wprA*, *htrA* and *htrB*, respectively (Fig. 1). BRB08 and BRB09 were then used to generate double mutants, namely BRB11 (*wprA*, *htrA*), BRB12 (*wprA*, *htrB*) and BRB13 (*htrA*, *htrB*). Finally, BRB11 was used to generate a triple mutant, BRB14 (*wprA*, *htrA*, *htrB*). The authenticity of their deletions was confirmed by sequencing (Supporting Information Table 3).

© 2013 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

3.2 Expression of PA

Together with the lethal and oedema factors, *B. anthracis* PA is a component of the anthrax toxin that, on its own, provides protective immunity against anthrax. rPA was used to test the performance of the constructed strains because previous work has shown that, while this protein is synthesised and secreted well by *B. subtilis*, it is rapidly degraded by both quality control and feeding proteases [12, 23], and the use of the protease-deficient mutant WB600 [24, 25] significantly reduces the extent of this degradation. rPA was expressed from the expression/secretion vector pHT28pagA using the IPTG-inducible P_{grac} promoter [26]. pHT28pagA was used to transform six *B. subtilis* strains (168, BRB07, BRB08, BRB11, BRB12 and BRB14) to determine the effects of the various protease deficiencies on growth and rPA production. An overnight culture in Terrific Broth was used to inoculate 5 L of modified Spizizen's minimal medium to an OD_{600nm} of ~0.4. At an OD_{600nm} of ~12, the cultures were induced with IPTG (1 mM) and incubation continued, feeding with glucose and a mixture of yeast extract and phytone peptone. All of the cultures grew well prior to induction (Fig. 2), with specific growth rates (μ) of ~0.91 h^{-1} (range 0.86–0.96 h^{-1}) for 168(pHT28pagA), BRB07(pHT28pagA) and BRB12(pHT28pagA), and ~0.66 h^{-1} (range 0.64–0.69 h^{-1}) for BRB08(pHT28pagA), BRB11(pHT28pagA) and BRB14(pHT28pagA). During the ~3 h following induction the specific growth rate of all of the strains decreased significantly: BRB08(pHT28pagA) to ~0.49 h^{-1}, 168(pHT28pagA), BRB11(pHT28pagA) and BRB12(pHT28pagA) to ~0.365 h^{-1} (range ~0.36–0.37 h^{-1}) and BRB07(pHT28pagA) and BRB14(pHT28pagA) to ~0.285 h^{-1} (range ~0.27–0.30 h^{-1}). The observed reduction in growth rate following induction presumably reflects the high-level expression of *pagA* and the secretion of its product.

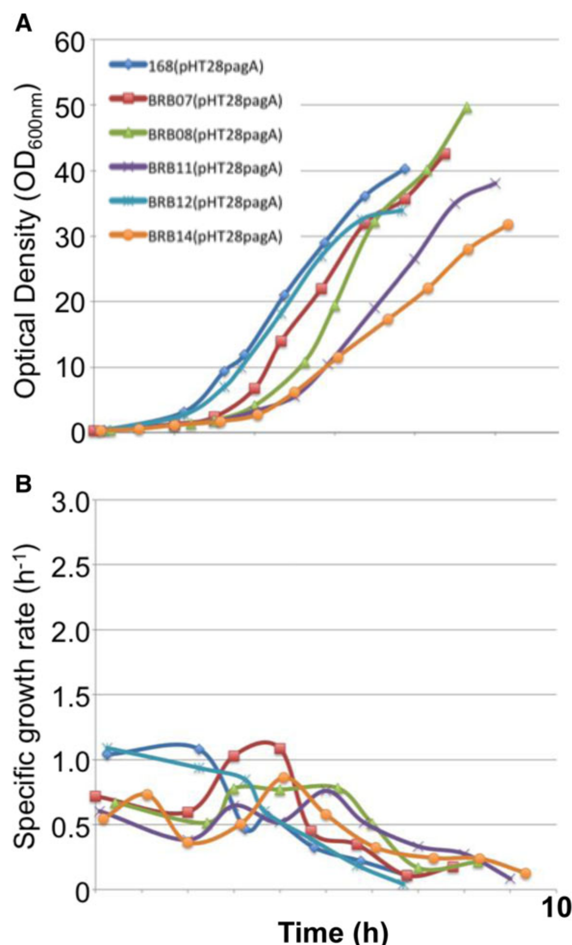

Figure 2. Growth profiles (A) and specific growth rates (B) of the wild-type and five protease-deficient mutants of *B. subtilis* containing pHT28pagA. Cultures were grown in a modified Spizizen's minimal medium to an OD_{600nm} of ~10 and then the *pagA* gene induced with 1 mM IPTG. Strains are *B. subtilis* 168 (pHT28pagA), BRB07(pHT28pagA), BRB08(pHT28pagA), BRB11(pHT28pagA), BRB12(pHT28pagA), BRB14(pHT28pagA).

3.3 Proteomic analysis of rPA production

Samples for proteomic analysis were taken at the point of induction and hourly for 5-h post-induction. Cells were removed by centrifugation and the supernatants analysed by SDS-PAGE (Fig. 3A) and Western blotting (Fig. 3B). SDS-PAGE clearly shows that rPA is rapidly degraded in the wild-type *B. subtilis* 168 strain, with only traces of this protein detected in a Western blot, using a polyvalent rabbit anti-rPA antibody. BRB07(pHT28pagA), lacking all seven feeding proteases, accumulates rPA in the culture medium to approximately 1 g/L, 5–6 h after induction with IPTG. Western blotting indicated the presence of a large number of degradation products. The remaining protease-deficient strains BRB08/11/12/14(pHT28pagA) showed a similar stabilisation and accumulation of rPA in the culture medium; while the production of rPA was highest in the BRB08-based strain, the presence of degradation products was decreased in the absence of either HtrA and/or HtrB (Fig. 3). As shown previously [27], wall-associated protein A (WapA) is stabilised in protease-deficient strains. The primary product of WapA (255 kDa) is processed by serine proteases to smaller proteins [28], identified as cell wall binding proteins CWBP200, CWBP105 and CWBP62 with corresponding molecular masses of 200, 105 and 62 kDa, respectively. The full-length and CWBP200 bands are identified in Fig. 3A. On the basis of size and its absence in the wild-type (Fig. 3), full-length WapA appears to be present in BRB08(pHT28pagA), BRB11(pHT28pagA) and BRB12(pHT28pagA). However, its absence in BRB14(pHT28pagA) indicated the presence of either intracellular serine proteases released by cell lysis, or the induction of another membrane/wall protease (see later).

The differential expression of proteins in the wild-type and protease-deficient strains following induction was analysed by 2D-PAGE. In the first place, we compared the patterns of proteins in the culture media of strains 168(pHT28pagA),

© 2013 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

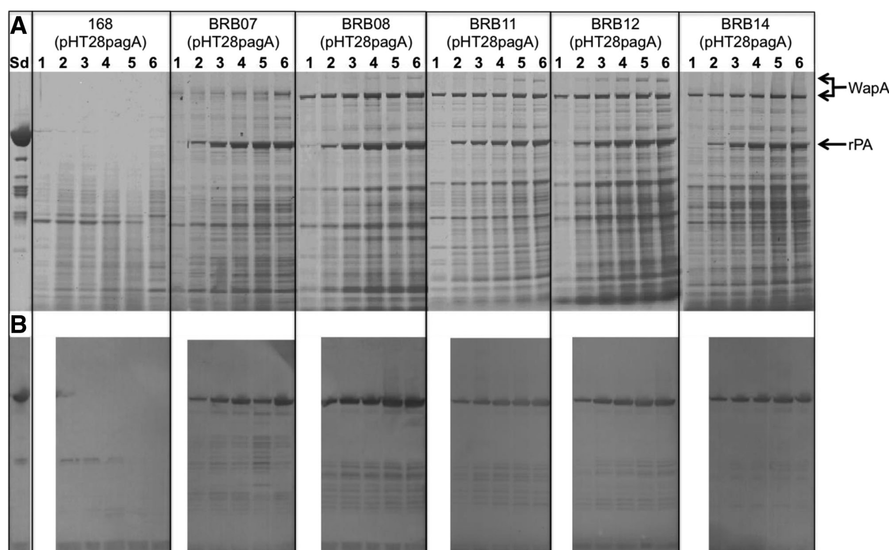

Figure 3. Anthrax PA detected by (A) SDS-PAGE and (B) Western blotting from fermentation supernatants of *B. subtilis* 168(pHT28pagA) and derivative strains BRB07(pHT28pagA), BRB08 (pHT28pagA), BRB11(pHT28pagA), BRB12(pHT28pagA) and BRB14 (pHT28pagA). Lanes are as follows: Sd, 3.7 μg rPA protein; (1) induction point; (2–6), sample points at 1–5-h post-induction. Only post-induction samples were subjected to Western blotting. The bands representing WapA (identified on the basis of size; [27, 28]) and rPA are indicated by arrows.

BRB07(pHT28pagA) and BRB14(pHT28pagA) with or without induction. Cultures were split at an OD_{600nm} of ~0.8 and one induced with IPTG (1 mM). The cultures were harvested 3 h later and supernatants proteins extracted for analysis. The growth yields of the induced cultures are lower than that of the non-induced cultures, with the growth rate of BRB14(pHT28pagA) being approximately half that of 168(pHT28pagA) and BRB07(pHT28pagA). To detect differences in the pattern of extracellular proteins between induced and non-induced cultures, the Delta2D software was used to pairwise overlay green (non-induced) and red (induced) false-coloured gels (Fig. 4). In the case of the protease-proficient strain, no full-length rPA was detected in the culture medium of the induced culture, and instead several lower molecular mass fragments of this protein were identified by MS (Fig. 4A, red spots). The absence of the seven feeding proteases in strain BRB07(pHT28pagA) significantly changed the profile of proteins in the culture medium, both because of the absence of the proteases themselves, but also because of their activity on other extracellular proteins such as WapA and WprA [21, 27]. In the presence of IPTG, a series intense spots corresponding to full-length rPA was detected (Fig. 4B). However, despite the absence of feeding proteases, several lower molecular mass spots corresponding to fragments of rPA were detected, pointing to the activity of the quality control proteases. In the case of BRB14(pHT28pagA), while full-length rPA was detected towards the top of the gel of the induced culture, the amount was significantly lower than that observed in BRB07(pHT28pagA; Fig. 4B). In part, this reflects the reduced culture density at harvesting. Significantly, the BRB14(pHT28pagA) culture medium did not contain prominent fragments of rPA.

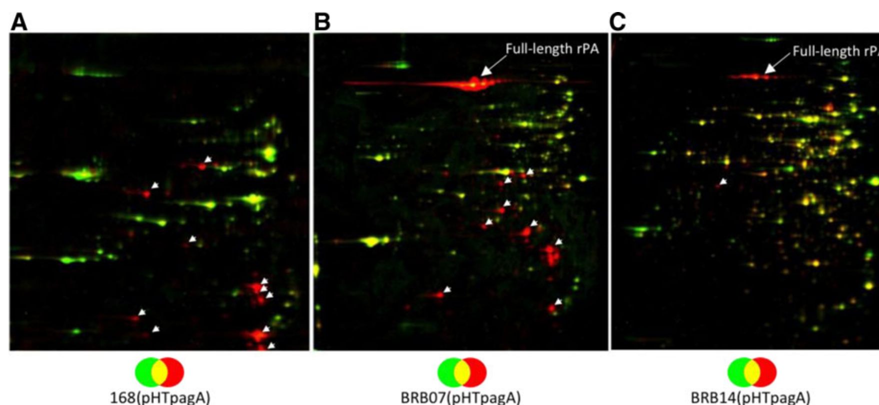

Figure 4. The extracellular proteome of shake flask cultures analysed by 2D PAGE. Cultures of 168(pHT28pagA), BRB07(pHT28pagA) and BRB14(pHT28pagA) were grown in LB medium to an OD_{600nm} of 0.8, split into two and IPTG (1 mM) added to one of the culture pairs. Incubation was continued for a further 3 h and, after removal of the cells, the proteins in the culture medium were subjected to 2D PAGE. The images from the non-induced (green) and induced (red) cultures were false coloured and overlaid. (A) 168(pHT28pagA), (B) BRB07(pHT28pagA) and (C) BRB14(pHT28pagA). The arrows indicate degradation products of rPA, as determined by MS.

© 2013 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

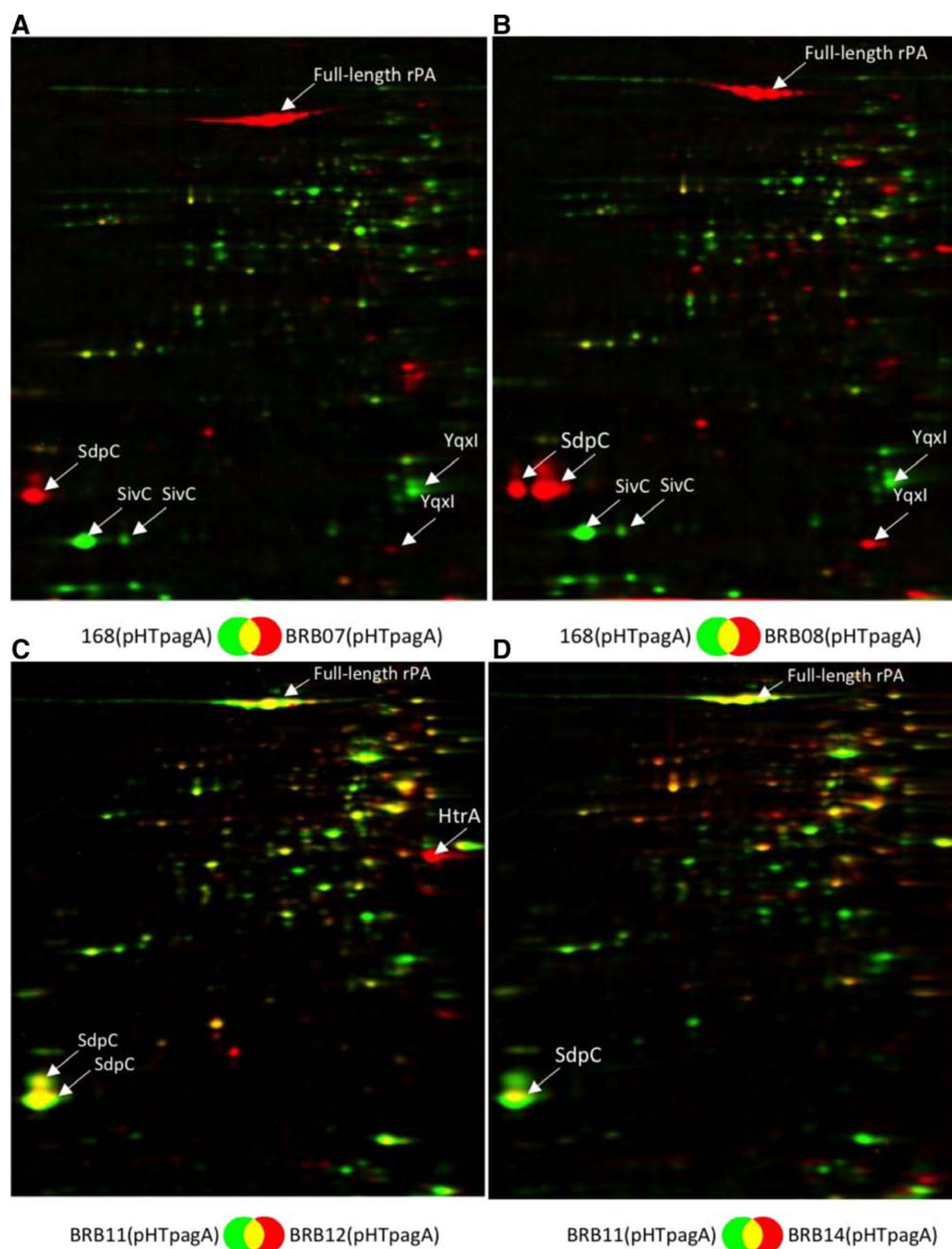

Figure 5. The extracellular proteome of cultures grown in an industrial fermenter analysed by 2D PAGE. Cultures of 168(pHT28pagA), BRB07(pHT28pagA), BRB08(pHT28pagA), BRB11(pHT28pagA), BRB12(pHT28pagA) and BRB14(pHT28pagA) were grown to an OD_{600nm} of 10 and induced with IPTG (1 mM). Incubation was continued for a further 4 h and, after removal of the cells, the proteins in the culture medium were subjected to 2D PAGE. The images were false coloured and overlaid in pairwise combinations. (A) 168(pHT28pagA)/BRB07(pHT28pagA), (B) 168(pHT28pagA)/BRB08(pHT28pagA), (C) BRB11(pHT28pagA)/BRB12(pHT28pagA) and (D) BRB11(pHT28pagA)/BRB14(pHT28pagA).

The production of rPA by the wild-type and protease-deficient strains under industrial fermentation conditions was also compared. Four litres of a defined rich medium were inoculated to an OD_{600nm} of ~0.3 and then induced at an OD_{600nm} of ~10.0 and supernatant samples for 2D-PAGE analysis taken 4 h after induction with IPTG (1 mM; Fig. 5A and B). Comparison of the extracellular proteins of 168(pHT28pagA) with either BRB07(pHT28pagA) or BRB08(pHT28pagA) showed that both of the protease-deficient strains produced full-length rPA but slightly different patterns of fragments (Fig. 5A and B, unlabelled red spots). Interestingly, the amount of SdpC, a secreted toxin

© 2013 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

that kills non-sporulating cells by collapsing the proton motive force and inducing autolysis, increased significantly, presumably because it was stabilised in the protease-deficient strains (Fig. 5A and B). SdpC is a member of the AbrB, Rok and Spo0A regulons. In contrast, the amount of a second secreted protein, SivC (YlqB), was decreased in the protease-deficient strains (Fig. 5A and B). SivC, a member of the AbrB and SigB regulons, is involved in inhibiting Spo0A activation and thereby the initiation of sporulation [29].

When the extracellular proteomes BRB11(pHT28pagA) and BRB12(pHT28pagA) lacking HtrA and HtrB, respectively, were compared, the main difference was the detection of HtrA in BRB12(pHT28pagA; Fig. 5C). We did not detect HtrB in the culture medium of BRB11(pHT28pagA), either because its concentration was too low and/or because unlike HtrA it was not released from the cell. The extracellular proteomes of BRB11(pHT28pagA) and BRB14(pHT28pagA) were very similar excepting for the presence in the latter strain of some additional proteins (Fig. 5D).

3.4 Characterisation of BRB14

The three HtrA-like proteins encoded by *B. subtilis*, HtrA (YkdA), HtrB (YvtA) and HtrC (YyxA/YycK), have N-terminal membrane-spanning domains and C-terminal extracytoplasmic serine protease domains. The expression of *htrA* and *htrB* is co-regulated as part of the CssR secretion stress regulon [30]. In the absence of either *htrA* or *htrB*, there is a compensatory upregulation of the other, indicating that they are likely to have similar roles in maintaining the quality of secretory proteins at the membrane-wall interface. In contrast, *htrC* is a member of the WalR cell-wall stress regulon, implying that its role is distinct from that of HtrA and HtrB [31].

Previous studies showed that an *htrA*, *htrB* double mutant exhibited greatly reduced viability and generated small mucoid colonies that spawned faster growing colonies with normal morphology [30, 32]. In contrast, the *htrA*, *htrB* double mutant BRB14(pHT28pagA) generated via BRB07 was stable and had a mean generation time (MGT) before induction of ~64 min, similar to that of the *htrA* mutant BRB11(pHT28pagA). However, following induction, its MGT increased to ~154 min, significantly longer than of the induced BRB11(pHT28pagA; ~115 min). The relatively rapid growth rate of BRB14(pHT28pagA) prior to induction was unexpected, implying either that the *htrA*, *htrB* double mutation is better tolerated in a multiply protease deficient background, BRB14 had acquired a suppressor mutation or *htrC* was induced to compensate for the lack of *htrA* and *htrB*. To address this issue, we sequenced the genome of BRB14(pHT28pagA) to identify potential mutations that might account for its increased stability. The sequence, based on 503 015 reads with an average 20-fold coverage, was assembled against the genome sequence of the parental strain *B. subtilis* 168 using the CLC Genomics Workbench 5 (CLCbio, Denmark). In

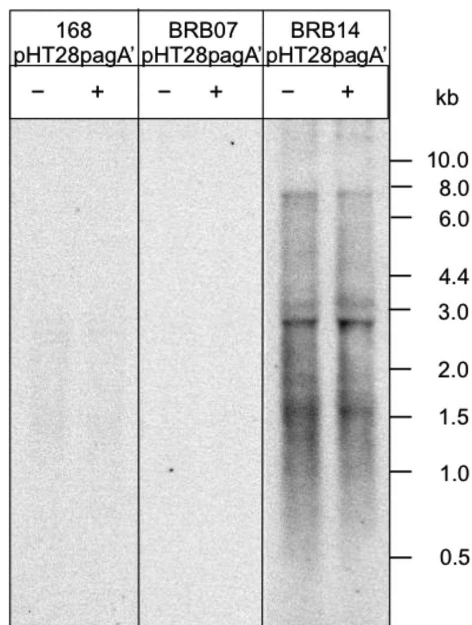

Figure 6. Northern blot analysis of *htrC* expression. The expression of *htrC* in cultures of 168(pHT28pagA), BRB07(pHT28pagA) and BRB14(pHT28pagA) with and without induction. Cultures were grown to an OD_{600nm} 0.8 in LB and split into two. IPTG (1 mM) was added to one of the culture pairs and incubation continued for 30 min. Total RNA was separated on a 1.2% agarose gels, blotted onto a nylon membrane, and hybridised with an *htrC* RNA probe.

addition to sequence variations associated with the deleted protease genes (Supporting Information Table 3), we identified single nucleotide polymorphisms (SNPs) in the following 11 genes: *amyE* (P28S), *argB*, *dnaE* (L1105W), *sacX*, *yfiG* (T152I), *yitF* (G368S), *yitQ* (S152L), *yitS* (T161*), *ywcH* (Y82C) and *ywlB* (R134Q). The SNPs in *argB* and *sacX* did not alter the amino acid encoded by their respective codons, while the protein encoded by *yitS*, putatively involved in fatty acid metabolism or transport, was truncated at the SNP site. The proteins encoded by *amyE* (α-amylase), *argB* (*N*-acetylglutamate 5-phosphotransferase), *dnaE* (α-subunit of DNA polymerase III), *yfiG* (D-chiro-inositol transport protein) are not known to be involved in secretion stress, while the functions of the proteins encoding *ywlB*, *yitF*, *yitQ* and *ywcH* are unknown. *yitQ* and *yitS* are located either side of the *nprB* gene, and we presume that the SNPs in these genes are associated with the generation of the Δ*nprB* mutation.

Because none of the SNPs in BRB14 appear to account for its stable phenotype, we compared the expression of the *htrC* gene in 168(pHT28pagA), BRB07(pHT28pagA) and BRB14(pHT28pagA). The strains were grown in LB medium to an OD_{600nm} of 0.8, split into two and IPTG (1 mM) added to one of the pairs of cultures. Total RNA extracted after 3 h was used in a Northern blot to monitor *htrC* expression (Fig. 6). These data clearly show that, irrespective of *pagA*

© 2013 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

induction, *htrC* is upregulated in BRB14(pHT28pagA) but not in 168(pHT28pagA) or BRB07(pHT28pagA). Whilst *htrC* is not induced by secretion stress, the absence of *htrA* and *htrB* leads to cell-wall stress, a response mediated by the WalRK signal transduction pathway [33].

4 Discussion

Bacillus subtilis secretes proteins efficiently into the culture medium and at a high concentration. However, the fate of these proteins is, to a large extent, dependent on their compatibility with the numerous extracytoplasmic proteases active at the membrane/wall interface, within the wall itself and in the culture medium. To reflect their physiological roles, these proteases have been categorised as either quality control or feeding proteases. Native *B. subtilis* exoproteins are generally either resistant to these proteases or are processed specifically into bioactive fragments (e.g. WprA and WapA) that are refractile to further degradation. In the case of heterologous proteins, two factors influence their behaviour and stability: the speed with which they fold in the highly charged environment on the *trans* side of the membrane (since unfolded proteins are more susceptible to proteolysis) and the presence of protease sensitive sites at the surface of a correctly folded protein [3, 4, 20, 21].

Previous studies have shown that the deletion of genes encoding extracytoplasmic proteases can improve the productivity of *B. subtilis* for both native and heterologous proteins [17]. Many of these strains were generated by allelic exchange of uncharacterised strains using antibiotic resistance markers as selective agent, and sequencing has shown that this approach leaves significant portions of their coding sequences (data not shown). As a result, such strains are not suitable for the production of therapeutic proteins. To address this limitation, we have generated a series of strains with precisely defined deletions, starting with a strain from an authenticated source. DNA sequencing confirmed the authenticity of each deletion (Supporting Information Table 3). We then used rPA as a model protein to demonstrate the effectiveness of the mutants because previous studies had shown that rPA was extremely susceptible to post-translocational degradation. The *pagA* gene was modified to replace its native signal sequence with that from the *B. licheniformis amyL* gene, and was expressed from the IPTG-inducible P_{grac} promoter.

By comparing the production of rPA by the wild-type and the various protease-deficient strains, it is clear that the biggest impact on stability occurred when the feeding proteases (*nprB, aprE, epr, bpr, nprE, mpr, vpr*) were inactivated. The inactivation of these genes had little or no impact on growth rate and yield compared with the wild type, even following the induction of *pagA*. Inactivation of *wprA*, encoding wall protease A, reduces the degradation of a variety of proteins, either as they emerge from the Sec translocase or in the cell wall [21, 34–36]. BRB08(pHT28pagA; Δ*wprA*) shows improved production of rPA over that of the feeding protease strain, confirming the role and activity of this quality control protease (Fig. 3). However, the MGT of BRB08(pHT28pagA) was ~30% longer than that of either the wild type or the strain deficient in the feeding proteases. The primary product of *wprA* (preWprA) is 894 amino acids in length (96.3 kDa), including a 31-residue signal peptide. The mature protein (863 residues) is processed into three proteins, CWBP23 from the N-terminus, CWBP52 from the C-terminus and a ~18 kDa linker peptide. Only CWBP23 and CWBP53 are detectable in the cell wall and the culture medium [27]. Although no activity has been ascribed to CWBP23, it is likely to be a propeptide involved in facilitating the folding of CWBP52 into an active serine protease.

Transformants in which either or both *htrA* and *htrB* were deleted exhibited normal colony morphologies and did not generate faster growing variants. Prior to induction, BRB11(pHT28pagA) lacking HtrA, grew more slowly (MGT ~65 min) than BRB12(pHT28pagA; MGT ~44 min) lacking HtrB. However, following induction both had a similar MGT of ~108 min, presumably reflecting the secretion stress associated with the high-level production and secretion of rPA. In the absence of HtrB, a band corresponding to an increase in HtrA production was clearly visible when the 2D-PAGE gels of BRB11(pHT28pagA) and BRB12(pHT28pagA) were overlapped (Fig. 5C). However, a spot corresponding to HtrB was not detected, either because it is not secreted from the surface of the membrane or its concentration is too low to be detected [27]. The importance of the HtrA and HtrB became apparent when the expression of *htrC* was monitored. The absence of these proteases leads to a marked increase in *htrC* expression, indicating an accumulation of misfolded secretory proteins at the membrane-wall interface that interferes with cell-wall biosynthesis and activates the wall stress induced WalR operon [30–32].

The genomic structure and secretory behaviour of the strains described here have been defined by a combined genomic and proteomic analysis, and provide a platform for the production of secreted recombinant proteins that complies with regulatory standards. They extend the range of strains available for biomanufacturing and synthetic biology. The role of the quality control proteases in influencing cell-wall synthesis, and the resulting effects on growth rate and the induction of the cell-wall stress regulon have also been investigated. To expand their utility to a greater range of proteins, further investigation of the intracellular bottlenecks in the secretion of heterologous proteins from *B. subtilis* is required.

We thank Dr. J. Gray for technical support with the mass spectrometry analyses. This work was supported by a U.K. Department of Trade and Industry LINK Applied Genomics Grant (APPGEN 79), a European Union Sixth Framework Grant (LSHC-CT-2004-503468) and an Engineering and Physical Sciences Research Council Synthetic Biology Grant (EP/J02175X/1).

The authors have declared no conflict of interest.

© 2013 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

5 References

[1] Tjalsma, H., Bolhuis, A., Jongbloed, J. D. H., Bron, S., van Dijl, J. M., Proteomics of protein secretion by *Bacillus subtilis*: separating the "secrets" of the secretome. *Microbiol. Mol. Biol. Rev.* 2000, *64*, 515–547.

[2] Harwood, C. R., Cranenburgh, R. M., Bacillus protein secretion: an unfolding story. *Trend. Microbiol.* 2008, *16*, 73–79.

[3] Pohl, S., Harwood, C. R., Heterologous protein secretion by *Bacillus* species from the cradle to the grave. *Adv. Appl. Microbiol.* 2010, *73*, 1–25.

[4] Sarvas, M., Harwood, C. R., Bron, S., van Dijl, J. M., Post-translocational folding of secretory proteins in Gram-positive bacteria. *Biochim. Biophys. Acta*, 2004, *1694*, 311–327.

[5] Sung, B. H., Lee, J. H., Kim, S. C., in: Lee, S. Y. (Ed.), *Systems Biology and Biotechnology of Escherichia coli*, Springer, Netherlands, 2009, pp. 19–40.

[6] Westers, H., Dorenbos, R., van Dijl, J. M., Kabel, J. et al., Genome engineering reveals large dispensable regions in *Bacillus subtilis*. *Mol. Biol. Evol.* 2003, *20*, 2076–2090.

[7] Manabe, K., Kageyama, Y., Morimoto, T., Ozawa, T. et al., Combined effect of improved cell yield and increased specific productivity enhances recombinant enzyme production in genome-reduced *Bacillus subtilis* strain MGB874. *Appl. Environ. Microbiol.* 2011, *77*, 8370–8381.

[8] Morimoto, T., Kadoya, R., Endo, K., Tohata, M. et al., Enhanced recombinant protein productivity by genome reduction in *Bacillus subtilis*. *DNA Res.* 2008, *15*, 73–81.

[9] Kunst, F., Ogasawara, N., Moszer, I., Albertini, A. M. et al., The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*. *Nature* 1997, *390*, 249–256.

[10] Leenhouts, K., Buist, G., Bolhuis, A., ten Berge, A. et al., A general system for generating unlabelled gene replacements in bacterial chromosomes. *Mol. Gen. Genet.* 1996, *253*, 217–224.

[11] Harwood, C. R., Cutting, S. M., *Molecular Biological Methods for Bacillus*, John Wiley, Chichester, UK 1990.

[12] Bloor, A. E., Cranenburgh, R. M., An efficient method of selectable marker gene excision by Xer recombination for gene replacement in bacterial chromosomes. *Appl. Environ. Microbiol.* 2006, *72*, 2520–2525.

[13] Baillie, L. W. J., Moir, A., Manchee, R., The expression of the protective antigen of *Bacillus anthracis* in *Bacillus subtilis*. *J. Appl. Microbiol.* 1998, *84*, 741–746.

[14] Antelmann, H., Tjalsma, H., Voigt, B., Ohlmeier, S. et al., A proteomic view on genome-based signal peptide predictions. *Genome Res.* 2001, *11*, 14984–14502.

[15] Büttner, K., Bernhardt, J., Scharf, C., Schmid, R. et al., A comprehensive two-dimensional map of cytosolic proteins of *Bacillus subtilis*. *Electrophoresis* 2001, *22*, 2908–2035.

[16] Hellman, U., Wernstedt, C., Góñez, J., Heldin, C. H., Improvement of an "in-gel" digestion procedure for the micropreparation of internal protein fragments for amino acid sequencing. *Anal. Biochem.* 1995, *224*, 451–455.

[17] Wu, X. C., Ng, S. C., Near, R. I., Wong, S. L., Efficient production of a functional single-chain antidigoxin antibody via an engineered *Bacillus subtilis* expression-secretion system. *Biotechnology* 1993, *11*, 71–76.

[18] Wu, S. C., Yeung, J. C., Duan, Y., Ye, R. et al., Functional production and characterization of a fibrin-specific single-chain antibody fragment from *Bacillus subtilis*: effects of molecular chaperones and a wall-bound protease on antibody fragment production. *Appl. Environ. Microbiol.* 2002, *68*, 3261–3269.

[19] Westers, L., Westers, H., Zanen, G., Antelmann, H. et al., Genetic or chemical protease inhibition causes significant changes in the *Bacillus subtilis* exoproteome. *Proteomics* 2008, *8*, 2704–2713.

[20] Jensen, C. L., Stephenson, K., Jørgensen, S. T., Harwood, C. R., Cell-associated degradation affects yield of secreted engineered and heterologous proteins in the *Bacillus subtilis* expression system. *Microbiology* 2000, *146*, 2583–2594.

[21] Stephenson, K., Harwood, C. R., The influence of a cell-wall-associated protease on the production of α-amylase by *Bacillus subtilis*. *Appl. Environ. Microbiol.* 1998, *64*, 2875–2881.

[22] Darmon, E., Noone, D., Masson, A., Bron, S. et al., A novel class of heat and secretion stress-responsive genes is controlled by the autoregulated CssRS two-component system of *Bacillus subtilis*. *J. Bacteriol.* 2002, *184*, 5661–5671.

[23] Williams, R. C., Rees, M. L., Jacobs, M. F., Prágai, Z. et al., Production of *Bacillus anthracis* protective antigen is dependent on the extracellular chaperone, PrsA. *J. Biol. Chem.* 2003, *278*, 18056–18062.

[24] Wu, X. C., Lee, W., Tran, L., Wong, S. L., Engineering a *Bacillus subtilis* expression-secretion system with a strain deficient in six extracellular proteases. *J. Bacteriol.* 1991, *173*, 4952–4958.

[25] Baillie, L. W. J., Moore, P., Manchee, R. J., Development of a *Bacillus subtilis* based system for the expression of the protective antigen of *Bacillus anthracis*. *Salisbury Med. Bull.* 1996, *87*, 133–135.

[26] Nguyen, H. D., Phan, T. T., Schumann, W., Expression vectors for the rapid purification of recombinant proteins in *Bacillus subtilis*. *Curr. Microbiol.* 2007, *55*, 89–93.

[27] Antelmann, H., Yamamoto, H., Sekiguichi, J., Hecker, M., A proteomic view on genome-based signal peptide predictions. *Proteomics* 2002, *2*, 591–602.

[28] Foster, S. J., Molecular analysis of three major wall-associated proteins of *Bacillus subtilis* 168: evidence for processing of the product of a gene encoding a 258 kDa precursor two-domain ligand-binding protein. *Mol. Microbiol.* 1993, *8*, 299–310.

[29] Garti-Levi, S., Eswara, A., Smith, Y., Fujita, M. et al., Novel modulators controlling entry into sporulation in *Bacillus subtilis*. *J. Bacteriol.* 2013, *195*, 1475–1483.

[30] Noone, D., Howell, A., Collery, R., Devine, K. M., YkdA and YvtA, HtrA-like serine proteases in *Bacillus subtilis*, engage in negative autoregulation and reciprocal cross-regulation of *ykdA* and *yvtA* gene expression. *J. Bacteriol.* 2001, *183*, 654–663.

© 2013 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

[31] Fabret, C., Hoch, J. A., A two-component signal transduction system essential for growth of *Bacillus subtilis*: implications for anti-infective therapy. *J. Bacteriol.* 1998, *180*, 6375–6383.

[32] Hyyryläinen, H-l., Bolhuis, A., Darmon, E., Muukkonen, L. et al., A novel two-component regulatory system in *Bacillus subtilis* for the survival of severe secretion stress. *Mol. Microbiol.* 2001, *41*, 1159–1172.

[33] Bisicchia, P., Lioliou, E., Noone, D., Salzberg, L. I. et al., Peptidoglycan metabolism is controlled by the WalRK (YycFG) and PhoPR two-component systems in phosphate-limited *Bacillus subtilis* cells. *Mol. Microbiol.* 2010, *75*, 972–989.

[34] Kobayashi, G., Toida, J., Akamatsu, T., Yamamoto, H. et al., Accumulation of an artificial cell wall-binding lipase by *Bacillus subtilis wprA* and/or *sigD* mutants. *FEMS Microbiol. Lett.* 2000, *188*, 165–169.

[35] Lee, S. J., Kim, D. M., Bae, K. H., Byun, S. M. et al., Enhancement of secretion and extracellular stability of staphylokinase in *Bacillus subtilis* by *wprA* gene disruption. *Appl. Environ. Microbiol.*, 2000, *66*, 476–480.

[36] Bolhuis, A., Tjalsma, H., Stephenson, K., Harwood, C. R. et al., Different mechanisms for thermal inactivation of *Bacillus subtilis* signal peptidase. *J. Biol. Chem.* 1999, *274*, 15865–15868.

© 2013 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim